SPECIFICATION

Extraction and Utilization of Cell Growth-promoting Peptides from Silk Protein

TECHNICAL FIELD

[0001]

The present invention relates to peptides, from silk protein, excellent for promoting cell growth, a production method thereof, and application thereof to the fields of medicament, quasi drug, cosmetics, etc., as a material for skin care as well as to cell culture substrates as a biomaterial.

RELATED ART

[0002]

Since silk threads have been used as a surgical suture from the old days, silk protein is regarded as a biocompatible material, and new developments focusing attention on this property have recently become active for its new uses in various fields.

For example, silk threads are solubilized to form an aqueous silk protein solution, followed by conversion to powder by precipitation, drying, grinding, etc., for additives of cosmetics; the aqueous silk protein solution is made to a film-like material by casting on a plate and the like for cell culture bed or wound-covering and coating material; and the silk protein solution is made to a gel-like material for use in food and cosmetics. The developments of these uses are pursued.

[0003]

Such developing examples include, for example, Kokai (Jpn. unexamined patent publication) 62-000415, Kokoku (examined patent publication) 01-044320, Kokai (Jpn. unexamined patent publication) 01-254164, Kokoku (examined patent publication) 06-004679, Kokai (Jpn. unexamined patent publication) 11-139986, Kokai (Jpn. unexamined patent publication) 11-276876, Patent No. 2997758, Patent No. 2990239, Kokai (Jpn.

unexamined patent publication) 11-253155, Patent application 2002-230656, Patent application 2002-148849, Kokai (Jpn. unexamined patent publication) 2001-163899. (See patent literatures from 1 to 12)

[0004]

During the development of new silk materials, it has become apparent that silk protein possesses various functions such as cell growth promotion, antioxidation, germicidal action, alcohol digestion and anticoagulation of blood.

However, it has not yet been elucidated which site or structure in silk protein is responsible for those functions.

The present inventors paid attention to the cell growth function associated with silk protein and have pursued the development and study of its function with the aim of utilizing cocoon filaments or silk threads as a skin care material for wound-covering materials, cosmetics and improved materials of synthetic fiber after these threads are solubilized and then converted into powder, film, gel and the like (for example, see patent literatures 8, 9, 10, 11 and 12).

In the course of the above development and study, it was found that the H and L-chains of fibroin and sericin a component, both constituting silk protein, have a growth-promoting action on fibroblasts originating from normal human skin (for example, see patent literatures 13, 14, and 15).

[0005]

On the other hand, cocoon filaments are used as a fiber, besides the field of clothing, in the fields of, for example, medical care (surgical suture for surgery), cosmetics (puff) and the like by processing cocoon via various process steps into raw silk and further into silk fabrics, and it has recently been recognized that the molecular weight of silk protein decreases during these processes of cocoon and raw silk.

Moreover, also in the process step of converting cocoon filaments or silk threads into powder, film, gel or the like (particularly, the solubilizing step of cocoon filaments or silk threads), the molecular weight of silk protein was found

to decrease [H. Yamada et al., Material Science & Engineering C,14, p.41-46 (2001)], [Tubouchi Kouzou, Yamada Hiromi, Takasu Yoko: the Japanese Society of Sericulture Science academic journal, Vol. 71, no.1, P.1-5 (2002)] (See nonpatent literature, nonpatent literature 2).

[0006]

Silk proteins with molecular weight decreased by such processes show a broad band with a smear in a molecular weight range between 10,000 and 200,000 daltons on an electrophoretogram.

Those with less than 10,000 daltons are mostly removed during the steps of dialysis and the like, and in fact, their molecular weights are decreased up to amino acid and oligopeptide levels.

The silk proteins with lowered molecular weights were found to have a reduced growth-promoting activity for human cells or to inhibit the cell growth (see patent literature 11).

[0007]

In other words, undegraded fibroin and undegraded sericin are excellent for promoting cell growth, whereas complex and heterogeneous cleavage of peptide bonds by the treatment with acid, alkali, light, heat, etc., during the process steps of cocoon resulted in inhibiting cell growth along with reduction in their molecular weights.

Accordingly, for the purpose of utilizing the cell growth-promoting function of silk proteins, it is preferred to use undegraded fibroin or undegraded sericin, fibroin H-chain (molecular weight, ca. 350,000) or L-chain, sericin a (molecular weight, ca. 400,000) and the like in their undegraded states.

[0008]

However, these three components (particularly, the H-chain and sericin a) have large molecular weights, and the stability in keeping their properties constant during prolonged storage is low.

Further, the L-chain is contained in fibroin only at less

than 10% by weight and is little in quantity.

Furthermore, there is yet no report clarifying which portion of each of these three components possesses cell growth-promoting activity.

[0009]

[Patent literature 1]

Kokai (Jpn. unexamined patent publication) 62-000415

[Patent literature 2]

Kokoku (examined patent publication) 01-044320

[Patent literature 3]

Kokai (Jpn. unexamined patent publication) 01-254164

[Patent literature 4]

Kokai (Jpn. unexamined patent publication) 04-202435

[Patent literature 5]

Kokoku (examined patent publication) 06-004679

[Patent literature 6]

Kokai (Jpn. unexamined patent publication) 11-139986

[Patent literature 7]

Kokai (Jpn. unexamined patent publication) 11-276876

[Patent literature 8]

Patent No. 2997758

[Patent literature 9]

Patent No. 2990239

[Patent literature 10]

Kokai (Jpn. unexamined patent publication) 11-253155

[Patent literature 11]

Patent application 2002-230656

[Patent literature 12]

Patent application 2002-148849

[Patent literature 13]

Kokai (Jpn. unexamined patent publication) 2001-163899

[Patent literature 14]

Patent application 2001-180169

[Patent literature 15]

Kokai (Jpn. unexamined patent publication) 2002-128691

[Nonpatent literature 1]

H. Yamada et al., Materials Science & Engineering C, 14, P.41-46 (2001)

[Nonpatent literature 2]

Tsubouchi Kouzou, Yamada Hiromi, Takasu Yoko: The Japanese Society of Sericulture Science academic journal Vol. 71, No. 1, P.1-5 (2002)

[Nonpatent literature 3]

Tashiro Yutaka and Otsuki Eiichi, Journal of Cell Biology, Vol. 46, P1 (1970)

[0010]

The fibroin H-chain and the a component of sericin are excellent for promoting cell growth, while they have high molecular weights larger than 300,000 and tend to crystallize.

In comparison to these, the molecular weight of the fibroin L-chain is 25,000 and smaller than that of the H-chain or the a component, but it has a tendency to crystallize more easily than ordinary proteins.

In addition, the ratio of fibroin H-chain to L-chain by weight is about 13 (H-chain) to 1 (L-chain), and the percentage of the L-chain is very small.

Proteins having a high molecular weight and high crystallinity are unstable in aqueous solutions.

Furthermore, when medicaments or cosmetic additives containing an oily ingredient or the like are added to these proteins, gel formation may readily occur, turning to unstable physical properties, and their stability during prolonged storage (more than 1 year) is low.

[0011]

On the other hand, substances which have a molecular weight lower than silk fibroin H-chain or sericin a and are excellent for cell growth include growth factors for various cells, for example, fibroblast growth factor (FGF) having a molecular weight ranging from 17,700 to 19,000.

These factors are secreted from tumors and cancerous cells, or from rapidly growing cells, and some of them are used as a therapeutic agent for skin ulcer (Fiblast Spray, Kaken

Pharmaceutical Co., Ltd.), but their safety is concerned.

SUMMARY OF THE INVENTION

The present invention was accomplished to solve these problems.

Namely, an object of this invention is to obtain peptides excellent in safety, stability due to their relatively low molecular weights, and cell growth promotion, all of which are different from cell growth factors produced by abnormal cells such as tumor cells.

[0012]

In order to solve the above problems, the present inventors have diligently conducted research and succeeded now in identifying that peptide chains located at specific sites of silk fibroin have cell growth-promoting function, thus completing the present invention.

That is, the present invention is significant in the aspect (1) that peptide compositions excellent for promoting cell growth comprise partial peptides of one or more peptide chains selected from peptide chains forming noncrystalline portions constituting silk protein, where the partial peptides have specific amino acid sequences formed of four to forty amino acid residues.

More specifically, the present invention provides peptide compositions excellent for promoting cell growth comprising the partial peptides from one or more peptide chains selected from the respective peptide chains of the N-terminal portion (I), the noncrystalline portion (A) and the C-terminal portion (a) constituting the H-chain of silk fibroin from a domesticated silkworm, the peptide chain of the L-chain thereof, and the respective peptide chains of the N-terminal portion (I), noncrystalline portion (A) and C-terminal portion (a) constituting the silk fibroin from a wild silkworm belonging to the genus Antheraea such as Antheraea yamamai, where the partial peptides have specific amino acid sequences formed of 4 to 40 amino acid residues.

[0013]

Further, a second aspect (2) provides a peptide composition where the peptide chains with the above specific amino acid sequences have any of the following amino acid sequences from (1) to (8):

(1)	A-6-2	VITTDSDGNE
(2)	A-6-6	NINDFDED
(3)	SfHE	AASSVSSASSRSYDYSRRNVRKN
(4)	SfHA	GSSGFGPYVAHGGYSGYEYAWSSESDFGT
(5)	AfH1	YGWGDGGYGSDS
(6)	AfH5	DEYVDN
(7)	AfH6	VETIVLEEDPYGHEDIYEED
(8)	AfH7	DDGFVLDGGYDSE

[0014]

Still further, a third aspect (3) provides a peptide excellent for promoting cell growth containing any of the following amino acid sequences from (1) to (8):

(1)	A-6-2	VITTDSDGNE
(2)	A-6-6	NINDFDED
(3)	SfHE	AASSVSSASSRSYDYSRRNVRKN
(4)	SfHA	GSSGFGPYVAHGGYSGYEYAWSSESDFGT
(5)	AfH1	YGWGDGGYGSDS
(6)	AfH5	DEYVDN
(7)	AfH6	VETIVLEEDPYGHEDIYEED
(8)	AfH7	DDGFVLDGGYDSE

[0015]

Still further, a fourth aspect (4) provides a method of separating and obtaining peptides excellent for promoting cell growth from the noncrystalline portions by hydrolysis of undegraded silk protein from a domesticated silkworm or undegraded silk fibroin from a wild silkworm belonging to the genus Antheraea, and by subsequent molecular fractionation.

[0016]

Still further, a fifth aspect (5) provides a method of separating and obtaining peptides excellent for promoting cell growth from the noncrystalline portions in the above fourth

invention wherein the hydrolysis is conducted by using a dilute acid, hydroxylamine or a protease.

[0017]

Still further, sixth (6) and seventh (7) aspects provide a cell growth-promoting agent containing the peptide compositions described in the above (1) and (2) and the peptides described in the above (3).

[0018]

Still further, eighth (8) and ninth (9) aspects provide a cell adhesion agent containing the peptide compositions described in the above (1) and (2) and the peptides described in the above (3).

[0019]

Still further, tenth (10) and eleventh (11) aspects provide a wound healing promoting agent containing the peptide compositions described in the above (1) and (2) and the peptides described in the above (3).

[0020]

Still further, twelfth (12) and thirteenth (13) aspects provide a cosmetic material containing the peptide compositions described in the above (1) and (2) and the peptides described in the above (3).

[0021]

Still further, fourteenth (14) and fifteenth (15) aspects provide a cell culture substrate containing the peptide compositions described in the above (1) and (2) and the peptides described in the above (3).

[0022]

This invention has succeeded in providing novel peptides excellent for cell growth having specific amino acid sequences of molecular weights lower than 10,000, preferably ranging from 4,000 to 400, by separating and fractionating peptides from the noncrystalline portions of silk protein as well as by synthesizing peptides similar to such peptides.

These peptides may be used as biomaterials such as cell adhesion agent, cell growth-promoting agent, wound healing

promoting agent, material for skin care like cosmetics, etc., and cell culture substrate.

DESCRIPTION OF THE PREFERRED EMBODIMENTSÍ

[0023]

The peptides from the noncrystalline portions of silk protein excellent for promoting cell growth and the peptide compositions thereof have been obtained by the following procedures and also determined for their amino acid sequences.

[0024]

(1) Cocoon layer from a wild silkworm belonging to the genus Antheraea such as domesticated silkworm or Antheraea yamamai is degummed, then solubilized in an aqueous solution of LiSCN and centrifuged. The separated supernatant is recovered and dialyzed against water. The dialyzate is again centrifuged and the separated supernatant is recovered.

(2) To the purified supernatant which has been separated and recovered, a protease such as chymotrypsin is added and treated for hydrolysis.

(3) The supernatant (noncrystalline portion) of the solution after the hydrolysis treatment is separated from the precipitate layer (crystalline portion) and recovered.

(4) The supernatant (noncrystalline portion) is fractionated by reverse phase chromatography.

(5) Each fraction fractionated by the reverse phase chromatography is subjected to cell culture.

(6) The fraction excellent for cell growth is fractionated by molecular weight by means of gel filtration chromatography, and fractions (peptide chains) having a cell growth rate 2-fold higher than that of a control fraction are determined for their amino acid sequences.

(7) Peptides expected to show excellent cell growth promotion are designed and synthesized based on the obtained peptide.

[0025]

Thus, the present invention can allow separation and fractionation of peptides of molecular weights lower than

10,000, preferably ranging from 4,000 to 400, having excellent cell growth-promoting activity from the noncrystalline portions of silk protein, synthesis of peptides analogous to such peptides, and provision of these peptides as biomaterials such as cell adhesion agent, cell growth-promoting agent, wound healing promoting agent, material for skin care like cosmetics, etc., and cell culture substrate.

[0026]

The cell growth-promoting peptides originating from silk protein of the present invention mean the peptides which show a cell growth rate 2-fold higher than that of a control in which cells are cultured in the absence of an added peptide in the three-day cell culture test as described in Examples 1 to 3.

[0027]

Primary structure of fibroin from domesticated silkworm

In the case of domesticated silkworm, the silkworm spins silk protein at the time of spinning to spin a cocoon (composed of cocoon filament and a pupa). In the cocoon filament, there exist fibroin at its center and sericin at its periphery, and their existing ratio is known to be 70 to 80% (fibroin) to 20 to 30% (sericin).

Fibroin in cocoon filament (silk protein refers to a combination of fibroin and sericin or each individually) is ca. 370,000 in its molecular weight.

[Tasiro Yutaka and Otsuki Eiichi, Journal of Cell Biology, Vol, 46, P1 (1970)] (See non-patent literature 3)

[0028]

In fibroin having a molecular weight of ca. 370,000, molecules having a size of ca. 350,000 (H-chain) and a size of ca. 25,000 (L-chain) are bound to each other via S-S bond.

The fibroin H-chain has a very high molecular weight, while it is composed of repetition of analogous sequences of amino acid residues.

[0029]

According to Gen Bank accession no. AF 226688, the precursor protein of the H-chain is composed of the N-terminal

portion (I), the repetitive part of a crystalline portion (R) and a noncrystalline portion (A), and the C-terminal portion (a).

The repetitive part is repeated by twelve crystalline portions (from R01 to R12) through the mediation of eleven noncrystalline portions (from A01 to A11) therebetween.

[0030]

The crystalline portions are composed of a long repetition of a highly crystalline dipeptide (Gly-X). X is highly crystalline Ala(A) or additionally Ser(S) as the main component, and other minor components thereof include Tyr(Y), Val(V), and the like.

In particular, the repetition of GAGAGS or GA is abundant.

As the result, the sum of G and A in the crystalline portion exceeds 50% and amounts to more than ca. 70%.

[0031]

The peptides of A01 to A11 in the noncrystalline portions are similar to one another in their amino acid sequences, while small differences are found in each amino acid sequence.

Further, the sum of G and A in each noncrystalline portion (from A01 to A11) is less than 50%.

The N-terminal portion and the C-terminal portion are considered to be noncrystalline from their amino acid sequences and amino acid compositions, and similarly to A01 to A11, the sum of G and A is each less than 50%.

On the other hand, there exist some portions in which the sum of G and A exceeds 50% in partial peptides of ca. 6 to 10 residues within the noncrystalline portions.

[0032]

<Precursor of fibroin H-chain>

I-R01A01R02A02R03A03R04A04R05A05R06A06R07A07R08A08R09A09R10A10R11A11R12-a

[0033]

N-terminal portion: I

N-terminal portion (I) is the initial peptide portion and its amino acid sequence is as follows:

MRVKTFVILCCALQYVAYTNANINDFDEDYFGSDVTVQSSNTTDEIIRDASGAVIEEQ
ITTKKMQRKNKNHGILGKNEKMIKTFVITTDSDGNESIVEEDVLMKTLSDGTVAQSYV
AADA

GAYSQSGPYVSNSGYSTHQGYTSDFSTSAAV

[0034]

Crystalline portion: R01, R02, ..., R12

All of R01, R02, ... R12 are portions called crystalline portion, and the number of amino acid residues is more than 300 for each crystalline portion. It should be noted, however, that the number of amino acid residues of R12 is 54.

The sum of G and A in each of the crystalline portions (R01 to R11) exceeds ca. 70%.

[0035]

Noncrystalline portions: A01, A02, ..., A11

They are composed of 28 to 32 amino acid residues and are called noncrystalline portion (A).

Their amino acid sequences are as follows:

A01 GSSGFGPYVANGGYSRSDGYEYAWSSDFGT
A02 GSSGFGPYVAHGGYSGYEYAWSSESDFGT
A03 GSSGFGPYVANGGYSGYEYAWSSESDFGT
A04 GSSGFGPYVAHGGYSGYEYAWSSESDFGT
A05 GSSGFGPYVAHGGYSGYEYAWSSESDFGT
A06 GSSGFGPYVANGGYSGYEYAWSSESDFGT
A07 GSSGFGPYVANGGYSGYEYAWSSESDFGT
A08 GSSGFGPYVANGGYSGYEYAWSSESDFGT
A09 GSSGFGPYVNGGYSGYEYAWSSESDFGT
A10 GSSGFGPYVANGGYSGYEYAWSSESDFGT
A11 GSSGFGPYVANGGYSRREGYEYAWSSKSDFET

[0036]

C-terminal portion: a

The amino acid sequence of the noncrystalline portion on the C-terminal side is as follows:

AASSVSSASSRSYDYSRRNVRKNCGIPRRQLVVKFRALPCVNC

[0037]

For convenience and reference to understand the present invention, notation for amino acids or amino acid residues in

protein, charge characteristics thereof and the like are described in the following Table 10.

[Table 10]

1 character	3 character	Amino acid	1 character	3 character	Amino acid
A	Ala	alanine residues	M	Met	methionine
C	Cys	cystine	N	Asn	asparagine
D	Asp	aspartic acid	P	Pro	proline
E	Glu	glutamic acid	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

[0038]

Based on the side chain characteristics, amino acids are classified as four groups consisting of acidic amino acid, polar non-charged amino acid, non-polar amino acid and basic amino acid

(1) Acidic amino acid: E, D

(2) Polar non-charged amino acid: N, S, Q, Y, T, C, F

(3) Non-polar amino acid : A, G, V, P, L, I, W

(4) Basic amino acid: H, K, R

[0039]

The fibroin L-chain shown below is a noncrystalline portion, when compared to the amino acid sequences of the crystalline portions and the noncrystalline portions of the fibroin H-chain.

<Amino acid sequence in fibroin L-chain>

MKPIFLVLLVATSAYAAPSVTINQYSDNEIPRDIDDGKASSVISRAWDYVDDTDKSIA
ILNVQEILKDMASQGDYASQASAVAQTAGIIAHLSAGIPGDACAAANVINSYTDGVRS
GNFAGFRQSLGPFFGHVGQNLNLINQLVINPGQLRYSVGPALGCAGGGRIYDFEAAWD

AILASSDSSFLNEEYCIVKRLYNSRNSQSNNIAAYITAHLLPPVAQVFHQSAGSITDL
LRGVGNGNDATGLVANAQRYIAQAASQVHV

[0040]

On the other hand, the amino acid sequence of silk protein from the wild silkworm is different from that of the domesticated silkworm, while wild silkworms belonging to the genus Antheraea such as Antheraea yamamai, Antheraea pernyi, Samia cynthia ricini, Antheraea assama and Antheraea mylitta have almost identical amino acid sequences.

For fibroin of Antheraea yamamai, the portion consisting of a repetition of 10 or more alanine residues (A) alone is referred to crystalline portion, and the other portions besides this are referred to noncrystalline portion.

Compared to fibroin from the domesticated silkworm, fibroin from the wild silkworm belonging to the genus Antheraea has a smaller number of residues in each repetitive part of the crystalline portion and the noncrystalline portion.

The amino acid sequences of the noncrystalline portions of fibroin from Antheraea yamamai excepting the crystalline portions having 10 or more sequential alanine residues are shown below.

From the amino acid compositions, the N-terminal portion and the C-terminal portion are also noncrystalline portions.

[0041]

<Primary structure of noncrystalline portions of fibroin from Antheraea yamamai >

N-terminal portion: initial peptide

MRVTAFVILCCALQYATANNLHHHDEYVDNHGQLVERFTTRKHYERNAATRPHLSGNE
RLVETIVLEEDPYGHEDIYEEDVVINRVPGASSSAAAASSASAGSGQTIIVERQASHG
AGGA

[0042]

Noncrystalline portions:

AGAAAGAAAGSSARGG

SGFYETHDSYSSYGSGSSSAAAASSGAGGAGGGYGWGDGGYGSDS

GSGAGGRGDGGYGSGSS

RRAGHDHAAGSSGGGYSWDYSSYGSES

GSGAGGVGGGYGGGDGGYGSGSS
RRAGHDRAAGS
SGAGGSGGGYGWGDGGYGSDS
GSGAGRAG
GDYGWGDGGYGSDS
RQAGHERAAGS
SGAGGSGRGYGWGDGGYGSDS
GSGAGGAGGDYGWGDGGYGSD
GSGAGGAGGDYGWGDGGYGSDS
SGAGGAGGGYGWGDGGYGSDS
SGAGGAGGYGGYGSDS
SGAGGSGGGYGWGDGGYGSGS
GSGAGGVGGGYGWGDGGYGSDS
SGAGGRGDGGYGSGSS
GSGAGGAGGGYGWGDGGYGSDS
RRAGHDRAAGC
SGAGGTGGGYGWGDGGYGSDS
SGAGGSGGGYGWGDGGYGSNS
SGAGRSGGGYGWGDGGYSSDS
SGAGGSGGYGGYGSDS
GSGAGGVGGGYGWGDGGYGGYGSDS
GSGAGGVGGGYGRGDSGYGSGSS
GHGRSSGS
SGAGGSGGGYGWDYGSYGSDS
SSGAGGSGGGYGWDYGGYGSDS
GSGAGGSGGGYGWGDGGYGSDS
SRRAGHDRAYGAGS
GAGASRPVGIYGTDDGFVLDGGYDSEGS

[0043]

C-terminal portion:

SSSGRSTEGHPLLSICCRPCSHRHSYEASRISVH

[0044]

As to silk protein components excellent for promoting cell growth, an analysis was performed on which portion of each component is responsible for cell growth promotion in the present invention, disclosing that it was associated with the

noncrystalline portions.

The noncrystalline portions of fibroin mean the portions having the amino acid sequences described above.

Accordingly, the N-terminal portion and the C-terminal portion are contained in the noncrystalline portions.

[0045]

All of A01 to A11 (peptides of 28 to 32 residues) of the noncrystalline portions from the domesticated silkworm show similar excellent cell growth promotion, while the amino acid sequences of A01 to A11 are not identical.

The sequence of amino acid residues of A11 differs from that of each of other A01 to A10 within 8 residues out of its 32 residues.

Accordingly, even if there is ca. 30% or less difference in the sequence of amino acid residues, the cell growth-promoting activity is not influenced.

And, even if there is an increase/decrease in the number of amino acid residues within ca. 20%, the cell growth promoting-activity is not influenced.

However, it is preferred that the difference in the amino acid sequences is less than 50% to retain the cell growth-promoting activity.

[0046]

On the other hand, peptides having a very small number of amino acid residues may not express the cell growth-promoting activity.

Particularly when the number of amino acid residues is not more than two, the cell growth may be inhibited.

Therefore, peptides for the cell growth promotion are better to be peptides of from 4 to 40 residues, preferably from 6 to 32 residues.

Further, it was found that a partial peptide containing more acidic amino acid residues and/or polar non-charged amino acid residues and few basic amino acid residues in the noncrystalline portions is better for the cell growth promotion.

Particularly, acidic amino acid residues showed an excellent cell-growth promoting activity.

[0047]

The noncrystalline portions show an excellent growth-promoting activity as a whole, whereas there exist, as a part, some sequence portions of amino acids showing a low growth-promoting activity because the sum of G and A exceeds 55% and also abundant basic amino acids are contained.

Accordingly, partial peptides in which acidic amino acids and/or polar non-charged amino acids are present and basic amino acids are hardly present are separated and recovered from the noncrystalline portions of fibroin.

Among the recovered partial peptides, the peptides having 40 or less amino acid residues and showing a value of cell growth rate two fold higher than that of a control in which there is no added peptide in the cell culture test described in Examples 1 to 3 are designated as SDFGP.

A mixture of a plurality of peptides having 4 to 40 amino acid residues is also designated as SDFGP, as long as its cell growth rate shows 2-fold or higher activity than that of the control.

[0048]

For the above conditions, a partial peptide from the noncrystalline portions is required to fulfill the following items, which is readily inferred from the results shown in Table 6:

(1) The sum of the numbers of G and A relative to the total number of amino acid residues in a peptide is not higher than 55%. This is because a higher content of G and A is liable to form a crystal.

(2) The number of basic amino acid residues relative to the total number of amino acid residues in a peptide is not more than 25%.

(3) There exist acidic amino acids and/or polar non-charged amino acids.

When these conditions are not met, the cell growth may

be inhibited.

[0049]

In this connection, the properties of amino acids or peptides differ by the chemical structures of their side chains.

Although acidic amino acids are excellent for cell growth promotion, the length of the side chain differs between E and D, and thus, flexibility or conformation of the side chain and principal chain differs between them, thus contributing to dissimilar cell growth promotion.

Likewise, this is true for various amino acids referred to as polar non-charged amino acids or basic amino acids, and due to variations in physicochemical properties, the same cell promoting and cell inhibiting activities are not always exerted.

[0050]

Cells forming our body are largely divided into adherent cells and floating cells.

The adherent cells include skin cells, vascular cells, gland cells, and the like. The floating cells include blood cells and the like.

The growth process of adherent cells starts with adhesion and then proliferation, and thus may be roughly divided into adhesion and proliferation.

SDFGP of the present invention has a property of supporting both adherence and proliferation, and is relatively better for adherence when compared between them.

Being better for proliferation gives rise to abnormal proliferation of cells, and may be associated with a safety issue for prolonged use.

Accordingly, SDFGP of the present invention which is good for adherence, has proliferation promotion, and does not inhibit cell growth is excellent for skin care materials and biomaterials.

When partial peptides are separated and recovered from silk protein in practice, silk protein is cleaved with a

protease that cleaves specific peptide bonds or otherwise with a chemical serving the same purpose, and then, peptides that are formed of not more than 40 amino acid residues and belong to the noncrystalline portions are recovered.

In addition, it is also possible to design a peptide excellent for cell growth on the basis of the obtained peptides and then synthesize the peptide.

[0051]

1. Separation and recovery of peptides from silk protein

In order to separate and recover the noncrystalline portions of silk protein, a characteristic of the noncrystalline portions may be used.

Since the noncrystalline portions are readily soluble in water, a silk material may be immersed in near neutral water (pH 5.0 to 9.0).

However, peptides of the noncrystalline portions cannot be efficiently obtained merely by the immersion of the silk material.

Hence, specific peptide bonds of silk protein are actively cleaved.

Such a cleavage method of peptide bonds preferably employs a chemical substance, an enzyme or the like which actively cleaves specific peptide bonds.

This is described below.

[0052]

1) Raw material

For the domesticated silkworm, raw material used is silk protein with fibroin H-chain, L-chain and remaining sericin a component.

[0053]

For the wild silkworm (e.g., Antheraea yamamai, Antheraea pernyi, Samia cynthia ricini, Antheraea assama and Antheraea mylitta,and the like), raw material used is the one in which a band of the fibroin is observed on an electrophoretogram as clearly as that of the fibroin H-chain from the domesticated silkworm.

The raw material may be individually fibroin and sericin, or those existing together.

[0054]

Thus, the raw material for the present invention may include all of cocoon filaments, raw silk, silk fabrics and knits, silk threads (fibroin fiber), remaining threads thereof or fibers, powder, film and the like with the use thereof as a raw material, and protein fiber material (silk material) spun by silkworm species such as domesticated silkworm and wild silkworm.

It should be noted that these materials have to contain the fibroin H-chain and sericin a component of domesticated silkworm and their corresponding components of wild silkworm.

Since the fibroin H-chain is more vulnerable to degradation than its L-chain, a partially remaining H-chain indicates that the L-chain is mostly left undegraded.

[0055]

2) Solubilization of the material

The material of the above 1) to be solubilized by a neutral salt may be a degummed material, half-degummed material, undegummed material, or an intermediate material thereof.

It may be sericin fiber from silkworm cocoon.

An important point is that their silk protein is not degraded or the fibroin H-chain, the a component of sericin and their corresponding proteins remain partially undegraded after various processing steps.

Whether the H-chain, the a component and the like remain or not is confirmed by the presence of their respective corresponding bands on an electrophoretogram.

[0056]

The neutral salt to be used as a solubilizer for raw silk threads includes, for example, calcium chloride, copper ethylenediamine, sodium thiocyanate, lithium thiocyanate, lithium bromide, magnesium nitrate, and the like.

The neutral salt is preferably in an aqueous saturated solution or at a concentration not less than 50% saturation

[weight (g)/volume (ml)].

In the case where fibroin and sericin are contained, for example, cocoon filaments, undegummed material, half-degummed material, and the like are solubilized using the above neutral salt in a manner similar to that for silk threads.

[0057]

On the other hand, when sericin occupies 98% or more as in the case of sericin fiber from silkworm cocoon, the solubilization is carried out with 8 M urea within 10 min at 70 to 90 degrees C.

Moreover, sericin fiber from silkworm cocoon may be solubilized with 9 M LiBr within 30 min at room temperature (20 to 30 degrees C).

The conditions for solubilization may be varied according to the above conditions.

[0058]

At the step of solubilizing the silk material in a neutral salt solution, an alcohol such as methyl alcohol, ethyl alcohol and propyl alcohol may be added to the neutral salt.

When calcium chloride is used as the neutral salt, the solubilization is carried out at a temperature not higher than 94 degrees C, preferably in the range of from 75 to 85 degrees C.

When lithium bromide is used, the raw material is solubilized at a temperature lower than ca. 50 degrees C. Thus, depending on the neutral salt used, the conditions for solubilization vary, and a method in which the fibroin H-chain and the a component of sericin may remain or its corresponding method is employed for solubilization.

[0059]

During solubilizing silk material,

(1) the solubilization may be accelerated by stirring.

(2) the solubilization is difficult at a lower temperature.

Although a higher temperature may facilitate the solubilization, a vigorous lowering in molecular weight may take place.

The solution in which silk is solubilized with a neutral salt contains the neutral salt, alcohol, and so on besides fibroin or a mixture of fibroin and sericin.

From this solution, insoluble materials are first removed, and then low molecular weight compounds having a molecular weight lower than 5,000 are removed using a dialysis membrane or a dialysis equipment.

A solution of silk protein is obtained by such dialysis.

[0060]

3) Degradation by enzyme and recovery

In general, it is said that cleavage of protein with a protease takes place at specific peptide bonds, and that modifications of the side chains of amino acid residues tend not to occur since its cleavage is carried out under a mild condition.

Furthermore, complicated fragmentation arising from nonspecific cleavage of protein may be avoided.

[0061]

Such enzymes include lysyl endopeptidase, arginyl endopeptidase, chymotrypsin, papain, pepsin, rennin, pancreatin, elastase and the like.

When a protease is added to an aqueous solution of silk protein, the silk protein is cleaved between specific peptide bonds.

Among these, chymotrypsin is particularly preferred to separate the crystalline and noncrystalline portions.

[0062]

When the cleaved peptides from silk protein are mainly composed of fragments generated from the crystalline portions of silk protein, these are prone to aggregate, and precipitate upon aggregation (coagulation or crystallization).

Even if their aggregation hardly occurs, peptides originating from the crystalline portion tend to aggregate more readily, and the addition of alcohol (methyl alcohol, ethyl alcohol, etc.) and the like as a coagulant triggers aggregation beginning from the peptides derived from the

crystalline portions, and they precipitate out.

[0063]

After removal of the precipitate, the remainder is a solution of peptides from the noncrystalline portions.

For the removal of the precipitate, the solution containing the precipitate is centrifuged (1,000 to 10,000 G) to remove it.

The solution freed of the precipitate is an aqueous solution of peptides, originating from the noncrystalline portions, which have been split off from the noncrystalline portions.

Upon drying this solution, film-like or powder-like peptides are obtained.

It should be noted that, when alcohol is added to the aqueous solution of peptides generated from the noncrystalline portions, the peptides from the noncrystalline portions start to aggregate in turn and precipitate out as the alcohol concentration becomes higher.

The peptides from the noncrystalline portions may contain partial peptides from the crystalline portions as long as the latter are within ca. 50%.

[0064]

However, to obtain peptides excellent for promoting cell growth, the sum of glycine and alanine residues are preferred not to exceed 55%, even if the peptides are those from the noncrystalline portions.

Further, the number of basic amino acid residues is preferred not to be higher than 25% of that of the residues constituting the peptide.

On the other hand, larger numbers of acidic amino acid residues and non-polar charged amino acid residues are preferred.

Particularly, a larger number of acidic amino acid residues is better for cell growth promotion.

The drying is carried out by lyophilization or spray drying.

Even if the peptides from the noncrystalline portions are crystallized after drying, they are readily soluble in water owing to their lower molecular weights.

[0065]

2. Synthesis of fibroblast growth-promoting peptide (SDFGP) from silk protein

The SDFGP separated and recovered from silk protein includes the following peptides:

These are merely examples of SDFGP.

Further, it goes without saying that there are many SDFGP satisfying the conditions described in [0047].

A-6-2	VITTDSDGNE
A-6-6	NINDFDED
SfHE	AASSVSSASSRSYDYSRRNVRKN
SfHA	GSSGFGPYVAHGGYSGYEYAWSSESDFGT
AfH 1	YGWGDGGYGSDS
AfH 5	DEYVDN
AfH 6	VETIVLEEDPYGHEDIYEED
AfH 7	DDGFVLDGGYDSE

Even only a portion of the amino acid sequences of these SDFGP may serve as SDFGP showing excellent cell-growth promotion.

Further, these peptides may be repeated within 40 residues or connected to other peptides.

[0066]

However, a single amino acid state is not endowed with the cell growth-promoting function.

The number of amino acid residues required for cell growth-promoting function is not less than 4 residues.

On the other hand, its preferred number is 40 or less in view of the efficiency of peptide synthesis.

The synthesis of SDFGP mimics the amino acid sequences of the noncrystalline portions of silk protein, and the peptides which satisfy the conditions described in [0047] are synthesized.

The number of amino acid residues of a synthetic peptide

is from 4 to 40, preferably from 6 to 32.

In this case, its amino acid sequence is not necessarily the same as that of silk protein.

[0067]

For example, the amino acid sequences of the noncrystalline portions (A0 to A11) of the fibroin H-chain are not totally identical, while their growth promoting-activities are approximately the same, and all of them conform to SDFGP.

Accordingly, its amino acid sequence may differ about 30% or less, preferably differ 50% or less.

In this case, a larger number of acidic amino acids is better, and the presence of polar non-charged amino acids is preferred.

In contrast, the absence of basic amino acids is preferred.

[0068]

In other words, the amino acid residues in each SDFGP may be replaced with other amino acid residues.

In this case, the number of basic amino acid residues is designed so as to become 25% or less of that constituting peptide.

On the other hand, a larger number of acidic amino acids in a peptide (SDFGP) is preferred.

The whole amino acid residues may be composed of acidic amino acids and/or polar non-charged amino acids.

[0069]

Thus, DSDGDE from A-6-2, DEDEDE and EDEDED from A-6-6, SSESSE and YGGYEY from SfHA, DGGYGGD from AfH 1, DEYDEY from AfHS, YEEDYEED from AfHG, and the like, and further many more peptides such as EEEE, EEEEEE, EYEYEY, EEYEEY, YYYYYY, EGSEGS may become SDFGP.

It goes without saying that these peptides may be repeated or connected to other peptides as long as they are in the range of the conditions described above in [0047] and have a molecular weight of 10,000 or less, preferably in the range of 4,000 to

400.

In addition, the amino residues may be replaced with other amino acid residues.

[0070]

4. Use

The peptide sets obtained from the noncrystalline portions of silk protein and SDFGP are not only excellent for cell growth promotion but also easily water-soluble.

Further, their molecular weights are approximately lower than 4,000, and therefore their forms are stable in a dissolved state for prolonged period.

Still further, mixing various SDFGP may promote higher cell growth compared to a single SDFGP.

Therefore, a mixture of these is added to cell culture medium, lotion, milky lotion, cream, ointment, instillation, food, and the like.

Since the peptide sets and SDFGP of the present invention are excellent for promoting skin cell growth, those are excellent materials for skin care, cell culture, and further for improvements in other fields besides the above, for example, clothing fiber, cosmetic powder, resin, etc.

[Example 1]

[0071]

Cell growth activity of enzymic digests of silk fibroin

(1) Enzymic degradation of the noncrystalline portions of silk fibroin, and subsequent separation and fractionation

Cocoons of a domesticated silkworm were cut open to remove pupae, and cocoon layers (10 g) were immersed in 30 volumes of 8 M urea for 10 min at 90 degrees C to extract sericin.

The residue after the extraction was washed with water, dried, and prepared as fibroin.

[0072]

One g of fibroin was immersed in 10 ml of 9 M LiSCN to solubilize. To this, 10 ml of distilled water was added and centrifuged for 10 min at 3,000 rpm.

The supernatant was put into a semipermeable membrane tube and dialyzed against 50 volumes of water.

The dialysis was performed four times, changing external dialysis water every 30 minutes.

After the dialysis, the solution was again centrifuged, and then 0.1 M sodium dihydrogenphosphate (pH 8.5) was added to adjust its pH to 7 to 8.

When chymotrypsin amounting to 1/100 of fibroin was added to that solution and left for 4 hours at 40 degrees C, precipitates were formed.

[0073]

Proteins or peptides in the supernatant originating from the noncrystalline portion (A) of fibroin were designated as noncrystalline portion (A'), and proteins or peptides in the precipitates originating from the crystalline portion (C) were designated as crystalline portion (C').

The crystalline portion was washed with water, dissolved in 1 ml of 9 M LiSCN and dialyzed against 50 volumes of water using a semipermeable membrane tube as described above, and the amount of protein in the aqueous solution was determined.

The amount of protein in the supernatant was determined without any further treatment.

[0074]

(2) Coating on cell culture vessel

The concentrations of aqueous solutions of these crystalline portion (C') and noncrystalline portion (A') were each adjusted to 0.025% and 0.0025%, respectively, by adding 70% ethanol, and 1 ml each of the diluted solutions was put into dishes (35 mmφ, Falcon) made of polystyrene and then air-dried.

Only 1 ml of 70% ethanol was added to dishes for control.

[0075]

(3) Cell culture

The cells used were human skin fibroblasts (originating from adult normal skin) purchased (frozen) from Sanko Junyaku Co., Ltd.

The culture medium used was the low serum culture medium for human skin fibroblast growth purchased from Kurabo Industries Ltd. [10 ml of LSGS (low serum growth supplement) added to 500 ml of Medium 106S (basal medium for skin fibroblast)].

It should be noted that LSGS has a cell growth-promoting activity.

The medium was added at 2 ml per dish, and 80,000 cells were inoculated and cultured for 3 days.

[0076]

(4) Measurement of viable cell number with Alamer Blue dye

The medium at 2 ml per dish and Alamer Blue (Iwaki Glass Co.) at 0.1 ml per dish were added and cultured for 2 hours at 37 degrees C, and then the reduced amount of Alamer Blue dye calculated from the absorbances at 570 nm and 600 nm was correlated with viable cell number.

The growth of human skin fibroblasts in dishes coated with the crystalline portion and the noncrystalline portion compared to that of control (100%) having no silk protein component is shown in Table 1.

[0077]

The cell growth rates in dishes coated with the silk protein components showed higher values in all cases compared to the control.

Particularly, the growth rate of the noncrystalline portion (C') at 0.025% concentration was high. This portion is a mixture of SDFGP.

The growth rate of the crystalline portion at a higher concentration (0.025%) was worse than that at a lower concentration (0.0025%).

This is considered to be due to an inhibition of the cell growth by silk protein of the crystalline portion.

[Example 2]

[0078]

Amino acid sequence of cell growth-promoting peptides

It was found in Example 1 that the noncrystalline portion

of fibroin promoted the cell growth more actively than the crystalline portion thereof.

However, the noncrystalline portion fractionated in Example 1 is likely to be a mixture of peptides generated by the enzymic cleavage.

Hence, the noncrystalline portion was further separated to identify the site of cell growth promotion.

First, reverse phase chromatography was carried out to separate the peptides contained in the noncrystalline portion in Example 1 according to their differences in polarity.

The column used was RESOURCER RPCk 3ml.

The chromatography was conducted with 0.1% TFA (trifluoroacetic acid) in pump A and 0.1% TFA/90% acetonitrile in pump B at a gradient of from 0% of B to 75% of B within 0 min to 15 min.

[0079]

As the result, 6 peaks (A-1, ..., A-6) were confirmed.

Each peak was collected, dried with an evaporator, and redissolved in a small volume of buffer (PBS).

The concentrations of A-1 to A-6 were adjusted to 0.025% with 70% ethanol, respectively, and then 1 ml each was put in cell culture dishes (35 mmφ, Falcon) made of polystyrene and air-dried.

Control dishes contained only 70% ethanol at 1 ml, followed by air-drying.

Using these dishes, a cell culture experiment was conducted.

The method for cell culture was the same as that in [Example 1].

The growth rates of human skin fibroblasts in dishes coated with the peptide fragments from the noncrystalline portion are shown in Table 2.

Among the fragments from the noncrystalline portion, A-6 was excellent for growth, showing ca. 4-fold enhancement compared to the control.

[0080]

Next, separation of peptides contained in A-6, which showed the best cell growth-promoting activity, according to their molecular weights was performed on Superdex peptide HR10/30 (gel filtration chromatography).

As the result, 7 peaks, A-6-1 to A-6-7, were confirmed.

All were under 2,500 in their molecular weights.

Among the 7 peaks, peptides in sharp and distinct 5 peaks (A-6-2, A-6-3, A-6-4, A-6-6, A-6-7) were recovered, and each peptide was coated on cell culture dishes, whereinto the medium and cells were inoculated and cultured for 2 days, followed by the measurement of the cell growth.

The experiment for the cell growth was the same as that in [Example 1].

The measurement results of cell growth rates are shown in Table 3.

The growth rates of A-6-2 and A-6-6 after 2-day culture were 1.5 fold higher than the growth of the control, showing excellent cell growth promotion.

The growth rates after 3-day culture exceeded 2 fold.

[0081]

Subsequently, the amino acid sequences of A-6-2 and A-6-6 were analyzed on LF3000 Protein Sequencer of BI Technologies Japan Ltd., and their amino acid sequences were found to be as follows:

A-6-2 VITTDSDGNE

A-6-6 NINDFDED

Both A-6-2 and A-6-6 were peptides from the N-terminal side of fibroin.

The N-terminal portion is a noncrystalline portion based on the amino acid composition.

Next, the peptides of A-6-2 and A-6-6 were synthesized (contracted out to Hokkaido System Science Co., Ltd.), and physiological activities of the synthesized peptides were determined after 3-day culture as described above, confirming that these showed cell growth promotion as the extracts from silk protein did.

In addition, when the synthesized A-6-2 and A-6-6 peptides were mixed, its cell growth promotion was superior to that of each peptide present individually in the cell culture.

[Example 3]

[0082]

Cell growth rates of synthetic peptides

Synthetic peptides (contracted out to Hokkaido System Science Co., Ltd.), which were derived from a total of 12 sites based on the amino acid sequences of the fibroin H-chain of the domesticated silkworm and the fibroin of Antheraea yamamai, were measured for their cell activity.

[0083]

(1) Peptide synthesis

Partial peptides from 2 sites of the crystalline portions (SfHC-1, SfHC-2), and partial peptides from 2 sites of the noncrystalline portions (SfHE, SfHA) were picked up from the fibroin of the domesticated silkworm and synthesized.

Further, Ala(A)-repeating site (AfH0) of the crystalline portion and 7 partial peptides (AfH1 to AfH7) of the noncrystalline portions were picked up from the fibroin of Antheraea yamamai and synthesized.

The partial peptides from the fibroin H-chain of the domesticated silkworm were 4 kinds, and the partial peptides from the fibroin of Antheraea yamamai were 8 kinds (AfH0 to AfH7). Each amino acid sequence of these peptides is shown below.

[0084]

Partial peptides from fibroin H-chain of domesticated silkworm (4 kinds)

SfHC-1	GAGAGSGAGAGSGAGAGYGAGY
SfHC-2	GAGAGSGAASGAGAGAGAGAGT
SfHE	AASSVSSASSRSYDYSRRNVRKN
SfHA	GSSGFGPYVAHGGYSGYEYAWSSESDFGT

[0085]

Partial peptides from fibroin of Antheraea yamamai (eight kinds)

AfH 0 AAAAAAAAAA

AfH 1 YGWGDGGYGSDS

AfH 2 SGAGGSGGYGGYGSDS

AfH 3 GSGAGGRGDGGYGSGSS

AfH 4 RRAGHDRAAGS

AfH 5 DEYVDN

AfH 6 VETIVLEEDPYGHEDIYEED

AfH 7 DDGFVLDGGYDSE

[0086]

(2) Coating of synthetic peptides on dishes

About 1 mg each of the synthetic peptides was dissolved in 200 µl of PBS. The dissolved peptide solutions were diluted with 70% ethanol to adjust to 0.025% and 0.0025% concentrations, and 1 ml each of these solutions were put in dishes and dried.

SfHC-1, SfHC-2 and SfHE were difficult to dissolve, and therefore, 1 ml of 9 M LiSCN was added to dissolve.

The dissolved solutions were put into semipermeable membrane tubes and dialyzed against 100 volumes of water with changes of external water every 30 minutes, and then their protein amounts were confirmed by an absorbance at 275 nm.

SfHC-2 is free of tyrosine and the like, and therefore its absorbance measurement was not possible. Since SfHC-1 and SfHE were present approximately in the same amount as they were present before dissolution, SfHC-2 was presumed to be present in the same amount as well and diluted with 70% ethanol in the same way as that used for other peptides, put in dishes and dried.

Further, AfH0 was also hard to dissolve and seemed to be lost upon dialysis due to its small molecular weight, and therefore it was dissolved by stirring well after dilution with 70% ethanol, put in a dish and dried.

[0087]

(3) Cell culture

The cells used were human skin fibroblasts (originating from adult normal skin) purchased (frozen) from Sanko Junyaku Co. Ltd.

The culture medium used was the low serum culture medium for human skin fibroblast growth purchased from Kurabo Industries Ltd.

The medium was added at 2 ml per dish, and ca. 80,000 cells were inoculated and cultured for 3 days.

Then, 0.1 ml of Alamer Blue (Iwaki Glass Co.) was added and cultured for 2 hours at 37 degrees C, and the reduced amount of Alamer Blue calculated from the absorbances at 570 nm and 600 nm was correlated with viable cell number.

The method of these measurements for cell culture is the same as that in Example 1.

The results are shown in Tables 4 and 5.

[0088]

As to the fibroin from the domesticated silkworm, the partial peptides of the noncrystalline portions, SfHE and SfHA, were higher in the growth rate compared to those of the partial peptides of the crystalline portions, SfHC-1 and SfHC-2, and showed more than 2-fold higher values than the control value, indicating that the partial peptides of the noncrystalline portions or their mixtures are SDFGP.

[0089]

There was little activity in AfH0 which is a partial peptide of the crystalline portion of the fibroin from Antheraea yamamai, and the higher concentration (0.025%) was lower in the growth rate, suggesting an inhibition of the cell growth.

The synthetic partial peptides of the noncrystalline portion, AfH1 to AfH7, showed cell growth promotion with differences in their growth rates

For example, both of AfH3 and AfH6 contain one basic amino acid residue, respectively. Since AfH3 contains less acidic amino acid residues and AfH6 contains more acidic amino acid residues, AfH6 showed higher cell growth promotion.

Particularly, AfH1, AfH5, AfH6 and AfH7 showed excellent cell growth promotion, and all of these are SDFGP obtained by synthesis.

[0090]

As shown in Table 5, not all partial peptides derived from the noncrystalline portions become SDFGP with high growth rates.

As shown in Table 6, this is due to the differences in the chemical structures of side chains of the amino acids constituting each peptide.

[Example 4]

[0091]

Cell growth activity of synthetic peptides

Based on the results in Examples 1 to 3, synthetic peptides (contracted out to Hokkaido System Science Co., Ltd.) having acidic amino acids and polar non-charged amino acids, which were supposed to show a cell growth activity, as the main components were measured for their fibroblast growth-promoting activity.

The measurements for the cell growth activity were carried out by a method similar to that in [Example 1].

The cell culture was performed for 3 days, and the results obtained at a peptide concentration of 0.025 $\mu g/cm^2$ are shown in Table 7.

As to glutamic acid, four or more sequential residues were excellent for cell growth activity.

[Example 5]

[0092]

Promotion of cell adhesion and proliferation by synthetic peptides

The cell growth activities of synthetic peptides originating from silk protein, synthetic peptides having mainly acidic amino acids or polar non-charged amino acids, or the like were further measured in detail by dividing into cell adhesion and cell proliferation.

This method partly differs from the method in (3) Cell culture of [Example 1].

In the present cell culture, the cells used were human skin fibroblasts (originating from adult normal skin)

purchased (frozen) from Sanko Junyaku Co. Ltd.

The culture medium used was the low serum culture medium for human skin fibroblast growth purchased from Kurabo Industries Ltd. [Medium 106S 500 ml].

The medium 106S is a basal medium for skin fibroblasts.

LSGS (low serum growth supplement) was not used here.

In the measurement of adhesion, the cells floating in the medium at 5 hours after inoculation of cells into the medium were removed, and the viable cell number adhering to the bottom of a dish was measured.

In the measurement of proliferation, the viable cell number at 3 days after inoculation of cells into the medium was measured.

Besides the cell culture method, coating of peptides on cell culture dishes, measurement of viable cell number with Alamer Blue dye, and the like were carried out as described in [Example 1].

The results obtained for adhesion and those obtained for proliferation are shown in Tables 8 and 9, respectively, in comparison with the viable cell number of the control (100%) where no peptide was coated on dishes.

[0093]

Namely, Table 8 shows adhesion after culturing human skin fibroblasts for 5 hours in dishes coated with various peptides.

Here, the synthetic peptides are shown either by symbol or amino acid sequence.

In this case, the concentration of each peptide coated on dishes was 0.025 $\mu g/cm^2$.

The adhesion rate (%) in the case where no peptide was coated was set at 100.

[0094]

Table 9 shows the proliferation after 3-day culture of human skin fibroblasts in dishes coated with various peptides.

Here, the synthetic peptides are shown either by symbol or amino acid sequence.

In this case, the concentration of each peptide coated

on dishes was 0.025 μg/cm^2.

The growth rate (%) in the case where no peptide was coated was set at 100.

It should be noted that the viable cell number in the control in 3-day culture increased about 150% during the culture and the control value (100%) was based on the viable cell number after 3-day culture.

Accordingly, when there was no increase or decrease in the cell number after 3-day culture, the growth rate would become about 70%.

[0095]

[Table 1]

Growth rates of human skin fibroblasts in dishes coated with crystalline portion and noncrystalline portion

	Concentration	Growth rate (%)	Significant difference from control; Confidence rate (%)
Control		100±8.6*	
Crystalline portion (C')	0.025%	149±5.6	99.9
	0.0025%	174±8.8	99.0
Noncrystalline portion (A')	0.025%	259±10.3	99.0
	0.0025%	164±5.0	99.0

(* Standard deviation)

[0096]

[Table 2]

Growth of human skin fibroblasts in dishes coated with peptide fragments from noncrystalline portion

	Growth rate (%)	Significant difference from control; Confidence rate (%)
Control	100±15.0*	
A-1	124±6.0	< 95
A-2	171±3.5	99
A-3	148±4.5	95
A-4	215±12.0	99
A-5	269±13.0	99.9
A-6	389±12.2	99.9

(* Standard deviation)

[0097]

[Table 3]

Growth rates of human skin fibroblasts in dishes coated with 5 fragments obtained from the peptide chain (Table 2, A-6)

	Growth rate (%)	Significant difference from control; Confidence rate (%)
Control	100±6.1*	
A-6-2	153±2.7	99.9
A-6-3	139±15.2	98.0
A-6-4	105±12.2	< 95.0
A-6-6	159±6.6	99.0
A-6-7	124±4.3	99.0

(* Standard deviation)

[0098]

[Table 4]

Growth of human skin fibroblasts in dishes coated with synthetic partial peptides of fibroin of domesticated silkworm

	Concentration	Growth rate (%)	Significant difference from control; Confidence rate (%)
Control		100±1.5*	
SfHC-1	0.025%	194±2.3	99.9
	0.0025%	146±6.0	99.9
SfHC-2	0.025%	136±1.0	99.9
	0.0025%	116±8.9	95
SfHE	0.025%	243±3.9	99.9
	0.0025%	198±12.5	99
SfHA	0.025%	316±5.7	99.9
	0.0025%	321±6.2	99.9

(* Standard deviation)

[0099]

[Table 5]

Growth of human skin fibroblasts in dishes coated with synthetic partial peptides of fibroin of Antheraea yamamai

	Concentration	Growth rate (%)	Significant difference from control; Confidence rate (%)
Control		100±26.5*	
AfH0	0.025%	100±7.4	< 50
	0.0025%	152±6.1	95
AfH1	0.025%	396±4.0	99
	0.0025%	179±13.5	98
AfH2	0.025%	196±9.4	99
	0.0025%	118±14.7	60
AfH3	0.025%	194±24.5	98
	0.0025%	111±9.0	< 50
AfH4	0.025%	133±8.7	80
	0.0025%	113±4.8	50
AfH5	0.025%	344±7.7	99.9
	0.0025%	192±11.3	99
AfH6	0.025%	264±9.0	99.9
	0.0025%	152±9.0	95
AfH7	0.025%	284±25.1	99.9
	0.0025%	155±5.3	95

(* Standard deviation)

[0100]

[Table 6]

Percentage of each amino acid group constituting synthetic peptides and cell growth rates

(The cell growth rates in Tables 4 and 5 are values obtained at higher peptide concentration (0.025%))

Partial peptide of fibroin of domesticated silkworm and Antheraea yamamai	Cell growth rate (%)	Number of residues in peptide	G + A		Basic amino acids	
			Number	(%)	Number	(%)
SfHC-1	194	22	18	82	0	0
SfHC-2	136	22	19	86	0	0
SfHE	243	23	3	13	5	4
SfHA	316	29	9	31	1	13
AfH0	100	10	10	100	0	0
AfH1	396	12	5	50	0	0
AfH2	196	16	9	56	0	0
AfH3	194	17	10	59	1	6
AfH4	133	11	5	46	4	36
AfH5	344	6	0	17	0	0
AfH6	264	20	1	35	1	5
AfH7	284	13	3	46	0	0

[0101]

[Table 7]

Cell growth activity of synthetic peptides

Amino acid or amino acid sequence of peptides	Cell growth rate (%)
E	69
EE	159
EEEE	231
EEEEEE	346
EEEEEEEEEE	254
YY	113
YYYY	156
YYYYYY	239
DEDEDE	322
EYEYEY	207
EEYEEY	212

[0102]

[Table 8]

Adhesion after 5 hour-culture of human skin fibroblasts in dishes coated with each peptide

Synthetic peptide	Adhesion rate (%)
SfHC-1	130
SfHC-2	138
SfHA	205
SfHE	190
AfH0	131
AfH1	169
AfH2	149
AfH3	174
AfH4	151
AfH5	234
AfH6	135
AfH7	153
DEDEDE	229

[0103]

[Table 9]

Growth after 3 day-culture of human skin fibroblasts in dishes coated with each peptide

Synthetic peptide	Growth rate (%)
SfHC-1	71
SfHC-2	97
SfHA	159
SfHE	126
AfH0	102
AfH1	94
AfH2	71
AfH3	69
AfH4	96
AfH5	108
AfH6	77
AfH7	89